The Proteases of Plants.

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SINCE the publication in these pages (June, 1903) of my last paper (1) on this subject, I have been continuing my investigations, and I have also come across several important papers by other observers, so that a considerable amount of further information has accumulated of which some account may now well be given.

Before discussing the new facts, I propose to indicate very briefly the desirability of somewhat modifying the current method of describing the phenomena of proteid-digestion, and to suggest a terminology more in harmony with the present state of knowledge. Hitherto the proteases of both plants and animals have been classified as 'peptic' or as 'tryptic,' in accordance with their general resemblance to either the pepsin or the trypsin of the animal body; and a digestion has been described as 'peptic' when it went no further than the conversion of the higher proteids into albumoses and peptones, and as 'tryptic' when the peptones formed were decomposed into non-proteid bodies such as leucin, tyrosin, &c. But with the discovery of erepsin by Cohnheim, this simple classification of the proteases has become inadequate, for erepsin is neither 'peptic' nor 'tryptic.' Of the two, it is more nearly allied with trypsin than with pepsin, inasmuch as it actively decomposes peptones: but it differs widely from trypsin in that it cannot peptonize the higher proteids, such as albumin and fibrin. It is, in fact, a representative of a new, third, class of proteases, which may be described as 'ereptic.'

Now as to the terms employed in describing the digestive process. The word 'proteolysis' is in common use, but not always in the same sense: it is sometimes applied to peptonization by pepsin: at other times, and more accurately, it is applied to the disruption of the proteid-molecule into non-proteid substances, and it is in this sense that I have used it in my more recent papers. But the most appropriate use of the word is its application to the sum-total of the processes involved in proteid-digestion, to all the changes determining the conversion of the higher proteids into such substances as leucin, tyrosin, &c. Accepting this connotation of

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'proteolysis,' the successive stages of the process may, I would suggest, be conveniently distinguished as—(*a*) *peptonisation*, the conversion of the higher proteids into albumoses and peptones; and (*b*) *peptolysis*, the decomposition of peptones into nitrogenous but non-proteid substances.

This terminology offers the prospective advantage of simplifying the classification of the proteases. But before attempting this, it is necessary to draw attention to a recent paper by Vernon (2) in which he announces the important discovery that the peptolytic activity hitherto attributed to trypsin is largely due to an ereptic enzyme associated with it. This enzyme, which may be distinguished as pancreato-erepsin, is not identical with the entero-erepsin found by Cohnheim in the small intestine, though it belongs to the same group, other members of which will no doubt be discovered in due time. The effect of this discovery is somewhat to alter the position of trypsin proper—that is, trypsin free from pancreato-erepsin—in a classification of the proteases, bringing its peptonizing activity into relatively greater prominence. Taking this into account, and neglecting the somewhat conflicting views as to the possible peptolytic activity of pepsin—which may, after all, be due to an associated erepsin hitherto undiscovered—the proteases of the animal body may be classified as follows:—

A. Actively peptonizing, but not at all peptolytic: pepsin.

B. Actively peptonizing and peptolytic: trypsin.

C. Feebly peptonizing, actively peptolytic: erepsins.

There is a question bearing upon the relation between trypsin and erepsin that requires special consideration. Trypsin, it is well known, forms tryptophane as one of the products of its peptolytic activity: but does erepsin produce this substance? It is not inconceivable that a peptolytic enzyme might produce leucin and tyrosin without, however, forming tryptophane; and if this were found to be true of any form of erepsin, it would afford a clear distinction between tryptic and ereptic proteases. It is unfortunate that, so far as I have been able to ascertain, the available information on this important point is not altogether conclusive. Cohnheim's account of the products of digestion by entero-erepsin conveys the impression that tryptophane was not among them: but it does not appear that the presence or absence of this substance was made the subject of special investigation. On the other hand, Dr. Vernon informs me by letter that he has detected tryptophane in a digestion of peptone by entero-erepsin. For the present, at any rate, I accept the positive rather than the negative evidence, adopting the view that tryptophane is a product of peptolysis by erepsin as well as by trypsin.

I have not included the proteases of plants in this survey, as I propose to discuss their nature in the concluding section of this paper.

In dealing with the papers on proteolysis in plants, to which I have alluded, I will take first those relating to cases that I have not myself

examined. There is, to begin with, an elaborate investigation by Butkewitsch (3) into the digestive action of certain of the lower Fungi (*Aspergillus niger*, *Penicillium glaucum*, and species of *Mucor*, *M. stolonifer*, *M. racemosus*, *M. Mucedo*) upon proteids. The Fungi in question were cultivated, in previously sterilized vessels, on a substratum consisting of proteid matter (Witte-peptone or fibrin) either with or without cane-sugar, together with a small proportion of suitable mineral ingredients acidified with phosphoric acid. The duration of an experiment varied from five days to over a month. The results show that these Fungi can peptolyse Witte-peptone, with formation of leucin and tyrosin, and can proteolyse fibrin, thus confirming the observations of earlier observers such as Malfitano (4) and others. A remarkable feature of the proteolysis effected by *Aspergillus* was the formation of a large proportion of ammonia (NH_3), though it was much smaller in the presence than in the absence of cane-sugar in the culture.

There is, further, a laborious research by Weis (5) into the nature of the proteolytic enzymes of malt. The author recognizes that in the germination of barley both peptonization and peptolysis take place—or as he puts it, there is a 'phase pepsique' and a 'phase trypsique'—whence he infers the presence of two distinct proteases which he respectively terms peptase and tryptase. The peptic action is apparently rapid, and soon comes to an end, whilst the tryptic action is slower and continues until the complete decomposition of the products of the peptic stage. The tryptic action was found to be only slight, at most, in a neutral liquid; rapid in the presence of a small quantity of added acid (e.g. lactic acid 0·2%; HCl 0·04%), and much retarded by the addition of alkali. The author is of opinion that the effect of acid and alkali upon the activity of proteolysis is to be explained, in accordance with the views of Fernbach and Hubert (Comptes rendus, t. 131, 1900, p. 293), who regard the primary (acid) and secondary (basic) phosphates present in the malt-extract as determining the course of proteolysis, the former promoting, the latter retarding it.

The author found both the peptic and the tryptic activity of malt to be interfered with by certain antiseptics, such as thymol, chloroform, formol, whilst toluol had but a slight effect. In the paper already referred to (1) I also have drawn attention to the influence of antiseptics on proteolysis in the special case of papaïn.

It was further ascertained that the proteases of malt-extract could digest various vegetable proteids other than the glutin of wheat; such as its own proteids, proteids of ungerminated barley, of rye, and of oats, legumin, vegetable casein; as also, among animal proteids, the fibrin of ox-blood, whilst the action on egg-albumin was slight.

YEAST (*Saccharomyces Cerevisiae*).

In a previous paper (6) I expressed the opinion, as the result of a few experiments, that yeast contains a proteolytic enzyme which is active in neutral and in acid liquids but not in alkaline. At that time I had not seen the paper in which Hahn and Geret (7) have given a full account of their very thorough investigation of this subject: their results are of such interest that a brief *résumé* of them will not be out of place. They worked with the expressed juice of fresh yeast, a liquid that contains a considerable amount of proteid coagulable on boiling, and is also spontaneously coagulable on being kept in the incubator at 37°C. for two hours. They ascertained that this liquid digested fibrin within twenty-four hours; but their investigation was directed more especially to the self-digestion (autolysis) of the liquid. Their method of estimating digestive activity was the comparison of the weights of the coagulum obtained from a given quantity of juice before and after digestion. For instance, in one case the weight of coagulum obtained before digestion was taken as 100, the weight after autolysis for twenty hours was 9·1. By this means they ascertained (*a*) that the natural acid juice digests actively; (*b*) that its activity is diminished, though not to any great extent, by such antiseptics as chloroform, thymol, toluol, salicylic acid, and hydrocyanic acid (HCN); (*c*) that it is increased by the presence of neutral salts, such as NaCl 3%, KNO_3 1%, KNO_8 10%; (*d*) that it is increased by the addition of HCl from 0·05% up to 0·3%, 0·2% HCl being the optimum, and that it is diminished in the presence of 0·5% HCl, and almost destroyed by HCl 1%; (*e*) that the activity is diminished by neutralization, and still more so by alkalinity of 0·2–0·5% NaHO. The inferences that they draw as to the nature of the proteolytic enzyme will be discussed in the concluding section of this paper.

Since the publication of the paper by Hahn and Geret, the only other contribution to the study of yeast-proteolysis is, so far as I am aware, that of Bokorny (8). He investigated the action of dried yeast, used in the solid form, upon various proteids either of animal or of vegetable origin: his experiments were made exclusively with liquids containing from 0·2–2% of added acid, chiefly phosphoric, without, apparently, any antiseptic, their duration varying from three hours to three days. The measure of digestive activity was the amount of the precipitate obtained on treating the concentrated digestion-liquid with excess of alcohol: the nature and relative quantity of the products was determined by dissolving the alcohol-precipitate in water, when any albumose present could be precipitated by saturation with ammonia sulphate or zinc sulphate, and any peptone by precipitation with phosphotungstic acid from the filtrate obtained after the separation of the albumoses.

The main conclusion at which Bokorny arrives is that the acid reaction is essential to the digestive activity of yeast, and that the degree of acidity has an important influence upon the character of the digestive process as indicated by the products: thus, when the acidity is less than 0·5 %, only a little albumose is formed, but a relatively large quantity of substances that are not precipitated by zinc sulphate or ammonia sulphate.

It can hardly be said that the paper adds material facts to existing knowledge of digestion by yeast, nor can the conclusion as to the relation between acidity and proteolysis be regarded as convincing. In the first place, the objection may be raised that no antiseptic was used; and though it may be urged that in many experiments the amount of acid present (0·5–1 %) was sufficient to prevent bacterial action, yet in those cases where the acidity was less strong, and the digestion prolonged (24–48 hours), the possibility of such action is obvious: in one case, indeed (Expt. 1), an offensive odour was noted and the development of mould. In the second place, no account is taken, in estimating the digestive products, of the proteids contained in the yeast itself. I have found that a watery extract of dried yeast, after boiling, filtering, and concentrating, yielded a mainly proteid precipitate with alcohol amounting to something like 20 % of the original weight (see p. 298): hence, when it is borne in mind that in Bokorny's experiments the weight of yeast employed amounted to 10, 20, or even 40 % of the proteid supplied for digestion, it is clear that the omission to take the yeast itself into account is a serious one. Finally, it is doubtful if any material amount of proteolysis was effected when the proportion of added acid was 0·5 % or more: for, as Hahn and Geret have pointed out, and as I have myself found, the digestive activity of yeast rapidly diminishes with increasing proportions of added acid (see p. 302).

I give now a selection of my experiments to illustrate the digestive activity of yeast under various conditions. I have employed fresh brewers' yeast, also yeast that I myself preserved in the dry state, but chiefly the dried yeast that is now obtainable as an article of commerce (prepared by the Granular Yeast Company Limited, London, E.C.), which is convenient to use, with the great advantage that it is possible to make a number of experiments with uniform material. The experiments include observations on self-digestion (autolysis), on the peptolysis of Witte-peptone, and on the proteolysis of fibrin. The test applied in the autolytic and peptolytic experiments was that for tryptophane, the presence of this substance being taken as evidence of peptolysis. When the experiments were comparative, the test had to be applied with certain precautions. Thus, in each set of observations, it was necessary to ascertain in some one case what quantity of chlorine-water had to be added to a given quantity of the digested yeast-liquid in order to produce a tryptophane-reaction

of maximum intensity; thus a standard of comparison for the other experiments in the same set was obtained. The quantity of chlorine-water required varies considerably, in relation, apparently, with the amount of tryptophane present: since an excess of chlorine destroys the reaction, it may be concluded that the more chlorine-water required, the greater the amount of tryptophane present. For instance, in an autolysis of a watery liquid containing 5 % dry yeast, I found that the addition of an equal volume of chlorine-water (say 5 cc. of each) gave the maximum tryptophane reaction, when the digestion had been short (say 4–6 hours): but when the digestion had been more prolonged (say 24 hours), it required twice the volume of chlorine-water to obtain a reaction as intense as that given as the result of the shorter digestion. It is also necessary to allow the tested liquid to stand for several minutes before estimating the intensity of the reaction, for it takes an appreciable time to develop. The liquid to be tested must, of course, have an acid reaction.

In the experiments with fibrin, the main object was the determination of peptonizing activity: accordingly the crucial test was the total disappearance of the fibrin, which was consequently supplied in small quantity (usually about 0·5 grm. to 100 cc. of liquid). The fibrin had been preserved in 50 % glycerin.

The dried yeast, previously to an experiment, was ground to a fine powder in a hand-mill, and was then thoroughly triturated in a mortar with the water necessary to prepare the required digestive liquid. The resulting mixture was then either used as it was, or it was filtered at a low temperature so as to prevent autolysis during the somewhat lengthy process, and the clear filtrate was employed. Toluol, to about 1 %, was found to be the most unobjectionable antiseptic, though I obtained good results with others, such as chloroform, sodium fluoride, and hydrocyanic acid. It is important to state definitely that in no case did the freshly-prepared yeast-liquid, whether mixture or extract, give any tryptophane-reaction, thus proving that the yeast used contained no tryptophane to begin with.

Autolysis.

The fact that, under certain circumstances, the yeast can digest its own proteids is a familiar one. My object in experimenting upon it was to ascertain something more definite as to the conditions determining the activity of autolysis, and as to the nature of the enzyme by which it is effected. The following experiments will give an idea of the method adopted and of the results attained.

Experiment 1. 1 grm. dried yeast placed in each of 3 bottles with 40 cc. distilled water: to No. 1 nothing further was added; to No. 2 was added 0·5 grm. precipitated chalk, to neutralize any free acid present; to No. 3, 0·1 grm. citric acid (= 0·25 %).

After about 20 hours in the incubator (temp. 38–40° C.) the tryptophane-reactions were: No. 1, marked; No. 2, strong; No. 3, faint.

A repetition of the experiment, using chloroform-water as the liquid, gave the same results.

A somewhat similar experiment was made with the object of ascertaining if so prolonged a period of digestion were necessary for autolysis.

EXPERIMENT 2. 40 cc. of an intimate mixture of 20 grms. ground yeast with 200 cc. of distilled water were placed in each of 5 bottles, to each of which toluol (1%) was added; the contents of the bottles were then varied as follows:—No. 1, nothing further added; No. 2, HCl to 0·05%; No. 3, HCl to 0·1%; No. 4, HCl to 0·2%; No. 5, Na_2CO_3 to 0·5%.

After 2½ hours in the incubator the tryptophane-reactions, on treating 5 cc. of the liquids with equal vol. of chlorine-water, were:—No. 1, very strong; No. 2, distinct; No. 3, marked; No. 4, distinct; No. 5, which was distinctly alkaline, strong: further addition of chlorine-water did not intensify the reaction in any case.

The following morning, after 20 hours more in the incubator, the tryptophane-reaction of Nos. 2 and 4 had become strong.

From this it appears that autolysis is a rapid process, a conclusion confirmed by another experiment in which one of two bottles, each containing 2 grms. dried yeast and 40 cc. distilled water, was kept for one hour in the incubator at 38°C., whilst the other bottle remained on the laboratory table at about 11°C. At the end of this time the contents of the former gave a distinct tryptophane-reaction, whilst those of the latter gave no reaction.

It appears, further, that autolysis can proceed within a wide range of alkalinity and acidity. The limits of this range were more nearly approached in the following experiment, which was of short duration:—

EXPERIMENT 3. 40 cc. of a mixture of 20 grms. dried yeast with 400 cc. distilled water, and toluol to 1%, were placed in each of 9 bottles, the contents of which were varied as follows:—No. 1, nothing further added; No. 2, added 2 grms. precipitated chalk; No. 3, added Na_2CO_3 to 0·5%; No. 4, added Na_2CO_3 to 1%; No. 5, added Na_2CO_3 to 2%; No. 6, added HCl to 0·05%; No. 7, added HCl to 0·1%; No. 8, added HCl to 0·2%; No. 9, added HCl to 0·5%.

After 4 hours in the incubator at 38° C., 5 cc. of each of these various liquids, treated with an equal volume of chlorine-water, after acidification with acetic acid where necessary, gave the following tryptophane-reactions:—Nos. 1 and 2, very strong; No. 3, which was neutral before acidification, gave a strong reaction, as did also No. 4, which was alkaline; No. 5, which also was alkaline before acidification, gave only a distinct reaction; Nos. 6 and 7 gave a strong reaction; No. 8 a distinct reaction; No. 9 no reaction.

The limit of acidity is here definitely indicated, the absence of the tryptophane-reaction proving that proteolysis did not take place in the presence of HCl added to 0·5%. The limit of alkalinity was not actually reached, though the retarding effect of 2% Na_2CO_3 was sufficiently

marked to justify the inference that a small further addition of alkali would arrest autolysis altogether.

As, however, the time of digestion in this experiment was short, it was necessary to ascertain whether similar results were obtainable with more prolonged digestion. In the case of HCl, a repetition of the foregoing experiment showed that no tryptophane-reaction was developed in a 0·5 % HCl liquid after digestion for 20 hours, and only a slight reaction after 72 hours. In yet another experiment, with 5 % yeast-mixtures containing respectively 0·2 %, 0·5 %, 0·8 %, and 1 % HCl, the tryptophane-reactions were—at the end of 24 hours' digestion—strong in the first, faint in the second, and none in the third or fourth; and at the end of 72 hours, strong in the first, distinct in the second, and still none in either the third or the fourth. Hence it appears that autolysis was much retarded by 0·5 % HCl, and altogether inhibited by 0·8 % or 1 %. In fact the limit of proteolytic activity in the presence of HCl lies between 0·5 % and 0·8 %, probably about 0·6 %, for a mixture containing 5 % yeast.

With regard to alkali, I found that a similar yeast-mixture, to which 2 % Na_2CO_3 had been added, gave no tryptophane-reaction after digestion for 72 hours. In this case I endeavoured to ascertain as nearly as possible the minimum time of exposure to the action of alkali required to arrest proteolytic action, by the following method:—

Experiment 4. 50 cc. of a 5 % yeast-mixture, with toluol, were placed in each of 3 bottles, Nos. 1, 2, and 3, to which Na_2CO_3 was added to the extent of 1 %, 2 %, 3 % respectively. The bottles were then placed in the incubator for a certain time. At the end of this time the contents of each bottle were divided into two equal portions, one of which was left alkaline, whilst to the other half HCl was added to slight acidity, and the 6 bottles were then returned to the incubator for any required number of hours, after which the tryptophane-reactions were compared.

In the decisive experiment of this kind, the 3 alkaline bottles were digested for 2 hours, when the tryptophane-reactions were, in No. 1, distinct; in No. 2, none; in No. 3, none. Half of the contents of each bottle having been acidified, the 6 bottles were further digested for 22 hours, when the reactions were:—

No. 1, still alkaline,	faint	acidified,	strong:
„ 2,	„ none	„	distinct:
„ 3,	„ none	„	none.

This experiment showed that digestion for two hours with 3 % Na_2CO_3 entirely destroyed proteolytic activity. A similar experiment, in which the period of exposure to this degree of alkalinity was only one hour, showed that this time did not suffice to destroy proteolytic activity, though it was much diminished.

The foregoing experiments were made with mixtures containing usually 5 % dried yeast: in none was the proportion less. It seemed

important to determine whether or not the results given by such a mixture apply equally to others containing less yeast, and it was found that they do not apply.

The following experiments were made with mixtures containing 2 % dried yeast:—

Experiment 5. *Acid.* 50 cc. of the mixture were placed in each of 3 bottles, Nos. 1, 2, and 3, acidified respectively with 0·1 %, 0·2 %, and 0·5 % HCl. After 3 hours in the incubator none gave any tryptophane-reaction; after 24 hours the reaction was faint in No. 1, none in either of the others.

Alkaline. In a similar experiment, in which the contents of the 3 bottles had been rendered alkaline by the addition of 1 %, 2 %, and 3 % Na_2CO_3 respectively, no tryptophane-reaction was obtained after digestion for 4 hours, or for 24 hours.

Here proteolytic activity was destroyed by 0·2 % HCl, as also by 1 % Na_2CO_3, degrees of acidity and alkalinity which produced no such effect in mixtures containing 5 % yeast. It may be concluded that there is a definite ratio between the amount of yeast present in a mixture and the amount of acid or alkali necessary to prevent autolysis.

On the evidence of the tryptophane-reaction, it results from the foregoing observations that autolysis is very active at the natural acidity of the yeast-mixture: anything more than a slight addition of either acid or alkali tends to diminish it. The acidity of yeast is partly due to the presence of organic acids; but not chiefly, for I have observed that it is impossible to neutralize a mixture or extract of yeast by adding excess of chalk. In view of the large proportion of phosphoric acid (about 50 %) and of potash (about 35 %) in the ash of yeast, it may be concluded that the acidity is mainly due to the presence of acid phosphate of potash. Naegeli (9) has in fact suggested that the cell-sap contains KH_2PO_4 and K_2HPO_4. Repeated digestions of mixtures to which excess of chalk had been added (see Experiment 3) have shown me that autolysis is even more active when the free organic acid present has thus been neutralized than at natural acidity. The conclusion to be drawn is that the most favourable degree of acidity is that afforded by the acid phosphates, a conclusion agreeing with that of Weis (see p. 291) in the case of malt. The influence of added acid on autolysis would appear to be, in accordance with the views of Fernbach and Hubert with regard to malt, that it is favourable so long as it merely suffices to convert the dibasic (K_2HPO_4) into monobasic (KH_2PO_4) phosphates, but unfavourable when free acid begins to accumulate. Similarly, the action of added alkali is favourable so long as it merely neutralizes any free acid present, but unfavourable when it begins to convert the monobasic into dibasic phosphates.

The study of autolysis necessarily involves the consideration of the proteids contained in the yeast-cell. I am not aware of any more recent

investigation in this direction than that of Naegeli (10), who stated the proteid content of yeast containing 8 % of nitrogen as follows:—

Ordinary albumin	36 %.
Gluten-casein, soluble in alcohol . . .	9 „
Peptone, precipitated by lead acetate . .	2 „

This statement is not altogether clear. The first item probably means that 36 % of the dry weight of the yeast consisted of coagulable proteid. The significance of the second item is doubtful: it is not impossible that it may really be albumose, or perhaps a mixture of albumoses and peptones. For some albumoses are relatively soluble in alcohol, precipitation only beginning with an alcoholic strength as high as 80 %; moreover, some of them possess the property, specially mentioned as characteristic of Naegeli's 'gluten-casein,' of readily giving off sulphuretted hydrogen when treated with caustic soda or potash. Again, peptone is to some extent soluble in alcohol when at all dilute; in fact one form (amphopeptone B) of it is soluble in 96 % alcohol. Finally, the substance described as 'peptone, precipitated by lead acetate,' is possibly not 'peptone' at all, since peptone proper (amphopeptone) is only partially precipitated by lead acetate: it is more probably one of the albumoses which are readily precipitated by this reagent.

In view of the rapidity with which autolysis took place, as indicated by the tryptophane-reaction, it seemed probable that the dried yeast used in my experiments contained albumoses or peptones, or a mixture of these, to begin with; and I have only so far investigated the proteids as to determine this point. A filtered watery extract was slightly acidulated and then boiled, when a precipitate of the coagulable proteids (albumin, &c.) was obtained. The filtrate was concentrated by evaporation and then treated with excess of alcohol, when a considerable precipitate was given. The precipitate was filtered off, dried, and dissolved in water on a filter; the solution was then saturated with ammonic sulphate, after the method of Kühne, in both alkaline and acid reaction, giving a considerable precipitate which consisted of albumoses. The filtrate, after appropriate treatment, still gave the biuret-reaction, indicating the presence of amphopeptone. Hence it is clear that the dried yeast contained both albumoses and peptones, the former in larger quantity than the latter. What still remains to be done is to determine the nature of the proteids that are coagulated on boiling.

Since there is evidence that the dried yeast contained albumoses and peptones, and since the test of proteolysis was the presence of tryptophane, my experiments do not throw light upon the peptonization of the higher proteids of the yeast in the course of autolysis. The conclusion to be drawn from them is that there is present in yeast a peptolytic enzyme which is most active at or near the natural acidity of a watery mixture or extract, which is due to the presence of acid phosphates.

Peptolysis.

Inasmuch as the foregoing experiments on autolysis were gauged by the tryptophane-test, they were essentially experiments in peptolysis. Nevertheless, I thought it necessary to institute experiments as to the peptolytic action of yeast upon added albumoses and peptones, as contained in the substance known as Witte-peptone: the results, as might perhaps be expected, were similar to those of the autolysis-experiments.

In the first place it was ascertained that a filtered watery extract of yeast was always peptolytically active, however short the period of extraction might be, even when the quantity of yeast used was small; and further, that peptolysis was rapidly effected. The following experiment, in which the period of extraction was limited to the time necessary for filtration, illustrates both these points:—

EXPERIMENT 1. 2 grms. of the dried yeast were extracted on a filter with 100 cc. of distilled water containing 1 % toluol: within an hour 50 cc. of liquid were obtained, to which 0·5 grm. Witte-peptone was added, and were then placed in the incubator. The liquid gave no tryptophane-reaction.

After digestion for one hour the liquid gave a marked tryptophane-reaction.

Here, notwithstanding the short duration of both extraction and digestion, a dilute extract gave unmistakable evidence of peptolytic activity.

The next experiment was made with the object of demonstrating the effect upon peptolysis of various strengths of acid and alkali.

EXPERIMENT 2. 20 grms. of dried yeast were extracted with 400 cc. of toluol-water (1 %), and left to filter for several hours in a cold room. The filtered liquid, which gave a faint tryptophane-reaction, was distributed as follows:—40 cc. were put into a bottle (No. 1) without further addition; in the remainder of the liquid 10 grms. of Witte-peptone were dissolved, and 40 cc. of the solution were put into each of 8 bottles (Nos. 2–9): to No. 2, nothing more was added; to No. 3, added 2 grms. of precipitated chalk; to No. 4, Na_2CO_3 to 1 %; to No. 5, Na_2CO_3 to 2 %; to No. 6, Na_2CO_3 to 3 %; to No. 7, HCl to 0·1 %; to No. 8, HCl to 0·2 %; to No. 9, HCl to 0·5 %.

After 4 hours in the incubator the tryptophane-reactions were:—No. 1, distinct; No. 2, marked; No. 3, strong; all three being acid: No. 4, distinct; No. 5, faint; No. 6, none; all three being alkaline: No. 7, strong; No. 8, marked; No. 9, distinct.

After 25 hours in the incubator the reactions were:—No. 1, distinct; Nos. 2 and 3, strong; No. 4, marked; No. 5, distinct; No. 6, none; Nos. 7 and 8, strong; No. 9, marked.

These results are in general agreement with those of the corresponding autolysis experiment (p. 295).

It was further ascertained that here also the retarding or inhibiting effect of added acid or alkali was the more marked the more dilute the yeast-extract.

Experiment 3. *Acid.* 4 grms. dried yeast were extracted for several hours in the cold with 200 cc. toluol-water (1 %): the filtered liquid gave a faint tryptophane-reaction: 2 grms. of Witte-peptone were dissolved in the liquid, 50 cc. of which were then put into each of 3 bottles acidified as follows:—No. 1, HCl to 0·1 %; No. 2, HCl to 0·2 %; No. 3, HCl to 0·5 %.

After 3 hours in the incubator the tryptophane-reactions were:—No. 1, distinct; No. 2, faint; No. 3, none: after 29 hours they were—No. 1, marked; No. 2, distinct; No. 3, none.

Alkali. 50 cc. of an exactly similar yeast-extract, containing the same percentage of Witte-peptone, were placed in each of 4 bottles: to No. 1, Na_2CO_3 to 1 % was added; to No. 2, Na_2CO_3 to 2 %; to No. 3, Na_2CO_3 to 3 %; to No. 4, nothing.

After 2 hours' digestion the tryptophane-reactions were:—in Nos. 1, 2, 3, faint; in No. 4, marked: the reactions were the same after the bottles had remained in the incubator for 25 hours.

On comparing the results of Expt. 3, where the strength of the yeast-extract may be taken as 2 %, with those of Expt. 2, where the strength of the extract may be taken as 5 %, it appears that the retarding action of added acid and alkali was more marked in the former than in the latter: for instance, in the case of the 2 % extract, peptolysis was inhibited by the addition of HCl to 0·5 %, and by all strengths of added alkali; whereas the 5 % extract peptolysed actively with HCl 0·5 % and with 1 % Na_2CO_3. This is very much the same relation as that indicated by the corresponding autolysis-experiments: such differences as exist are due to the different chemical composition of the liquids in the two sets of experiments.

The fact that the retarding action of added acid and alkali is less marked in peptolysis than in autolysis is clearly brought out by a comparison of the results obtained by a peptolytic experiment on the same lines as the autolytic experiment (Expt. 4, p. 296), which had as its object to determine the effect of exposure to the action of alkali of different strengths for a short time, and showed that autolysis was inhibited by treatment for two hours with 3 % Na_2CO_3 liquid at 38°C.: complete inhibition was not produced in the peptolytic experiment under similar conditions.

Experiment 4. 10 grms. of dried yeast were extracted for several hours with 200 cc. toluol-water (1 %) and filtered: the filtered liquid gave faint tryptophane-reaction. 50 cc. of the liquid were placed in each of 3 bottles, to which Na_2CO_3 was added to 1 %, 2 %, 3 % respectively, and then the bottles were kept in the incubator for 2 hours. The contents of each bottle were then divided into 2 equal parts, in separate bottles, and one half was slightly acidified with HCl, whilst the other half remained alkaline: 0·2 grm. of Witte-peptone was added to each bottle, and a little more toluol.

After 24 hours' digestion the tryptophane-reactions were :—

	Na_2CO_3 1 %	2 %	3 %
Alkaline . . .	faint	faint	none
Acid	strong	strong	marked

24 hours later they were :—

Alkaline . . .	marked	faint	none
Acid	very strong	very strong	strong.

These experiments with Witte-peptone confirm the conclusion arrived at from the autolysis-experiments (see p. 298)—that yeast contains an actively peptolytic enzyme, most active at or near the natural acidity of the extract, becoming less active, to total arrest, on the addition of either acid or alkali. Further, they show that this protease can be very readily extracted with water, and that the peptolytic action of a watery extract is marked as well as rapid, even when (as in Expt. 1) the extract is dilute (2 %).

Peptonization.

Having ascertained that yeast is actively peptolytic, I proceeded to investigate its peptonizing capacity, the test being the complete disintegration of a small quantity of fibrin. The experiments were made with (*a*) solid yeast substance, (*b*) aqueous extracts, (*c*) extracts made with 2 % NaCl solution.

(*a*) *Experiments with solid yeast substance.* The first of these experiments was of a general character, with the object of ascertaining definitely if digestion of fibrin were effected by yeast, and how digestion would be influenced by added alkali and acid.

Experiment 1. In each of 6 bottles were placed 40 cc. distilled water and 5 grms. of partly dried brewers' yeast, with toluol as the antiseptic; 0·5 grm. of fibrin was added to each, and the bottles were severally treated as follows:—to No. 1, nothing further was added; to No. 2, 1 grm. chalk (reaction remained acid); to No. 3, Na_2CO_3 to 0·5 %; to No. 4, HCl to 0·04 %; to No. 5, HCl to 0·1 %; to No. 6, HCl to 0·2 %.

After 20 hours in the incubator the fibrin had not disappeared in any bottle, though in some it had diminished; 24 hours later it had disappeared in Nos. 1 and 2; 24 hours later it had disappeared in No. 3, whilst most of it remained in the others.

In a repetition of this experiment (omitting bottle 6), with 10 grms. of yeast (25 %) in each bottle, the fibrin disappeared in all the bottles within 48 hours.

These experiments show that yeast can digest fibrin; and that the activity of any given mixture, as also its resistance to the retarding action of added acid or alkali, depends upon the amount of yeast that it contains. These two points were then further investigated. The material used in the following experiments was the dried 'granular' yeast already mentioned.

With regard to the relation between the digestive activity of a mixture and the quantity of yeast contained in it, I found to begin with that 50 cc. of a mixture of chloroform-water with 1·25 % yeast did not digest 0·2 grm. fibrin in 70 hours. This relation, as well as the action of acid and alkali, is further determined in the following comprehensive experiment:—

EXPERIMENT 2. Mixtures were prepared of toluol-water (1 %) with 2·5 %, 5 %, 10 %, and 20 % dried yeast respectively. 40 cc. of each of these mixtures were put into each of 3 bottles, to one of which nothing was added, to the second Na_2CO_3 to 2 %, to the third HCl to 0·5 %, and 0·3 grm. fibrin to each bottle. After 22 hours' digestion the results were:—

	Added *nothing.*	*Na_2CO_3.*	*HCl.*
20 % bottles; fibrin	gone	gone	not gone
10 „ „ „	gone	gone	not gone
5 „ „ „	not gone in any:		
2·5 „ „ „	not gone in any:		
after further digestion for 25 hours—			
20 % bottles; fibrin	—	—	not quite gone
10 „ „ „	—	—	not gone
5 „ „ „	gone	not gone	not gone
2·5 „ „ „	gone	not gone	not gone
after further digestion for 28 hours—			
20 % bottles; fibrin	—	—	gone
10 „ „ „	—	—	not gone
5 „ „ „	—	not gone	not gone
2·5 „ „ „	—	not gone	not gone

24 hours later, when the experiment closed, the results were the same.

These results suffice to indicate the relation between the digestive action on fibrin of yeast-mixtures of different strengths, and the degree to which digestive activity is affected by fairly strong acid and alkali in each case. Those afforded by the bottles containing 0·5 % HCl are of special interest in relation to Bokorny's experiments, in which, as I have already pointed out (see p. 293), the conditions seem to have been such as to prevent any digestion at all of the added proteid. This criticism applies more particularly to those of his experiments (Nos. 1–8) in which the amount of yeast present was 5 %, the strength of acid 0·5–1 % H_3PO_4, and the proteid to be digested (ten times the weight of the yeast employed) the meat-residue from the preparation of Liebig's extract. The facts upon which I base this criticism are supported by other results, subsequently described, obtained in experiments with yeast-extracts. It is more difficult to criticize Bokorny's further experiments (Nos. 9–15), in which proteids of vegetable origin (prepared from Pea-flour, Soja-bean-meal, and Rape-cake) were the material to be digested, since the quantitative relations are not clearly stated: but, in view of the small amount of digestive products obtained,

and the possibility that a considerable proportion of these may be attributed to the relatively large quantities of dried yeast added, amounting to 30 % or more of the weight of the proteid to be digested, they appear to be open to the same objection as the others.

In all the foregoing experiments unboiled fibrin was used, so that a possible source of error was present. In order to eliminate this, some experiments were made in which the fibrin had been boiled for a few minutes. I found that 50 cc. of both a 10 % and a 20 % mixture of yeast with toluol-water digested 0·3 grm. fibrin in four days.

(*b*) *Experiments with aqueous extracts.* The foregoing experiments with solid yeast afforded no information as to the solubility of the digestive protease; so I had recourse to filtered watery extracts in the first instance. It was soon ascertained that active extracts can be obtained under suitable conditions. The following experiment proves this, and gives some indication of the effect of acidity and of alkalinity:—

EXPERIMENT 1. 12½ grms. of dried yeast were extracted (24 hours) with 250 cc. distilled water (yeast = 5 %). 50 cc. of the filtered liquid were put into each of 4 bottles, with 0·2 grm. fibrin, and treated thus:—to No. 1, nothing further added; to No. 2, Na_2CO_3 to 0·5 %; to No. 3, HCl to 0·05 %; to No. 4, HCl to 0·2 %.

After 21 hours' digestion in the incubator the fibrin in Nos. 1 and 3 showed diminution, in Nos. 2 and 4 it was not affected: 23 hours later it had disappeared in Nos. 1 and 3, but remained unaffected in Nos. 2 and 4.

In the next place I tested the digestive activity of stronger yeast-extracts, whether of natural acidity, or alkaline, or with added acid.

EXPERIMENT 2. Extracts of 10 grms. and of 20 grms. dried yeast with 100 cc. of toluol-water (1 %) were prepared and filtered. 30 cc. of each extract were put into each of 3 bottles with 0·3 grm. fibrin, one bottle of each having nothing added, another bottle having Na_2CO_3 added to 2 %, and the third bottle HCl to 0·5%.

After 27 hours' digestion in the incubator the condition of the fibrin was—

	Nat. acid.	*Na_2CO_3.*	*HCl.*
bottles 10 % yeast	gone	unaltered	unaltered
,, 20 % ,,	gone	attacked	gone
21 hours later it was—			
bottles 10 % yeast	—	unaltered	attacked
,, 20	—	attacked	—

48 hours later the fibrin was still unaltered in the 10 % extract with Na_2CO_3, and had not disappeared in either the 10 % extract with HCl or the 20 % extract with Na_2CO_3.

Hence it appears that both 10 % and 20 % watery yeast-extracts actively digest fibrin, and that, as might be expected, the latter are less affected by added acid and alkali than the former. Comparing the results of this experiment with those of the corresponding experiment (see p. 302)

with solid yeast, it is clear that the resistance of solid yeast to acid and alkali is greater than that of the filtered extracts.

The following experiment shows that a dilute and rapidly prepared watery extract has little or no action on fibrin :—

EXPERIMENT 3. 2 grms. of dried yeast were extracted on a filter with 100 cc. toluol-water (1 %), the whole process being completed in 2 hours. 50 cc. of the filtrate were put into a bottle with 0·2 grm. fibrin. The fibrin had not disappeared after digestion in the incubator for 5 days.

Having observed that the digestion of boiled fibrin took place in the presence of solid yeast, I made an experiment of this kind with yeast-extract, and with the same result: 60 cc. of a 20 % yeast-extract digested 0·2 grm. fibrin in 4 days.

(*c*) *Experiments with 2 % NaCl extracts.* It occurred to me that it should be possible to prepare yeast-extracts that would digest fibrin more actively than did the aqueous extracts, by the use of some solvent other than distilled water. I found such a solvent in a 2 % solution of common salt (NaCl): extracts made with this liquid, even when rapidly prepared, can be depended upon to digest fibrin, and are therefore specially suitable for the investigation of the digestive action of yeast upon this proteid.

The following experiment, with a 10 % yeast-extract, gives a general indication of the effect of various antiseptics and of HCl upon the digestion of fibrin :—

EXPERIMENT 1. 20 grms. of dried yeast were extracted for several hours on a filter with 200 cc. of 2 % NaCl solution. 25 cc. of the filtrate were placed in each of 6 bottles, treated respectively as follows:—No. 1, nothing further added; No. 2, added HCN to 0·2 %; No. 3, added NaF to 1 %; No. 4, added chloroform to 0·5 %; No. 5, added toluol to 0·5 %; No. 6, added HCl to 0·2 %: to each added 0·2 grm. fibrin.

After 26 hours in the incubator the fibrin had disappeared in all the bottles except No. 6, where it seemed to be quite unaltered.

The following experiment demonstrates the inhibiting action of added acid and alkali :—

EXPERIMENT 2. 40 grms. of dried yeast were extracted for several hours with 400 cc. of 2 % NaCl solution containing 1 % toluol. 40 cc. of the filtered liquid, with the addition of a little more toluol, were put into each of 9 bottles with 0·3 grm. fibrin: the further additions were—to No. 1, nothing; to No. 2, 2 grms. precipitated chalk; to Nos. 3, 4, 5, HCl to 0·1 %, 0·2 %, 0·5 % respectively; to No. 6, H_3PO_4 to 0·5 %; to Nos. 7, 8, 9, Na_2CO_3 to 1 %, 2 %, 3 % respectively.

After 5 hours in the incubator the fibrin in Nos. 1, 2, 3 showed signs of solution. After 24 hours it had disappeared in Nos. 1, 2, 3, and 7: it had not perceptibly diminished in any of the others, nor had it done so 72 hours later.

These results show that addition of Na_2CO_3 to 2 %, or of H_3PO_4 to 0·5 %, or of HCl to 0·2 %, inhibits the digestive action of a 10 % yeast-extract made with 2 % NaCl solution. On comparing them with those obtained in the corresponding experiments with 10 % solid yeast (p. 302), and with 10 % aqueous extract (p. 303), there is complete agreement as regards the effect of added acid, a matter of importance in relation to Bokorny's experiments; and as regards the effect of added alkali, the only preparation that withstood the action of 2 % Na_2CO_3 was that containing solid yeast.

The inhibiting effect of added alkali was further investigated by the method employed in the corresponding experiments in autolysis (p. 296) and peptolysis (p. 300).

EXPERIMENT 3. 10 grms. of dried yeast were extracted on a filter for 3–4 hours with 200 cc. 2 % NaCl solution containing 1 % toluol. 50 cc. of the filtrate were placed in each of 3 bottles, to which Na_2CO_3 was added to 1 %, 2 %, 3 % respectively; the bottles were then placed in the incubator for 2 hours. On being removed, the contents of each bottle were divided into 2 equal parts, one of which remained alkaline, the other being made slightly acid with HCl. There were then 6 bottles, 3 acid and 3 alkaline, each containing 25 cc. of liquid: to each was added 0·2 grm. fibrin and a little more toluol.

The 6 bottles were placed in the incubator, together with another bottle, No. 7, containing 25 cc. of the original extract, which had not been treated with Na_2CO_3, and 0·2 grm. fibrin.

After 20 hours' digestion the fibrin had disappeared in bottle No. 7 (natural acidity): but it had not undergone any apparent change in Nos. 1–6, nor had it done so after 48 hours' digestion.

Thus treatment for two hours with even 1 % of Na_2CO_3 sufficed to destroy the digestive activity of a 5 % yeast-extract made with salt-solution, a result that more closely defines the action of alkali than those of the preceding experiments, in which it was ascertained that neither a 5 % yeast-mixture (p. 302) nor a 10 % watery extract (p. 303) digested fibrin in the presence of 2 % Na_2CO_3.

But it has not yet been made clear what is the advantage of a NaCl extract over a watery extract of yeast. When the extracts are strong, say 10 %, the advantage is not very marked: the digestive action of both is vigorous and rapid, more especially when the period of extraction and filtration has been prolonged for many hours. When, however, dilute and rapidly prepared extracts are employed, the greater activity of the NaCl extract is most apparent, as the following experiment shows.

EXPERIMENT 4. 1 grm. of dried yeast was treated with 50 cc. toluol-water (1 %); 1 grm. of yeast was treated with 50 cc. of 2 % NaCl solution: the mixtures were at once filtered, so that the preparation of the filtered liquids did not last more than 2 hours. To each bottle 0·2 grm. fibrin was added, and then they were both put into

the incubator. After 20 hours' digestion the fibrin in the watery extract was unaltered, whilst that in the NaCl extract was much diminished, and 4 hours later had disappeared. The fibrin in the watery extract had not disappeared after digestion for 4 days longer.

In another experiment in which extraction was prolonged for several hours, the activity of stronger aqueous and 2 % NaCl extracts of yeast was compared. It was found that 30 cc. of a 10 % yeast NaCl extract digested 0·2 grm. fibrin within 18 hours, and the same quantity of 5 % yeast NaCl extract digested the fibrin in 24 hours; whereas the times of digestion by the corresponding aqueous extracts were 46 and 66 hours respectively.

The object of the next experiment was to ascertain in what way NaCl affects digestion. Does it directly promote it, or does it do so indirectly by dissolving out of the yeast something that distilled water fails to extract or extracts less completely?

EXPERIMENT 5. 3 grms. of dried yeast were extracted with 60 cc. of 2 % NaCl solution (toluol 1 %); 6 grms. were also extracted with 120 cc. of toluol-water: extraction and filtration occupied about an hour and a half. 40 cc. of the NaCl extract were put into a bottle (No. 1) with 0·2 grm. fibrin: of the aqueous extract, 40 cc. were put into a second bottle (No. 2), and other 40 cc. into a third bottle (No. 3), to which NaCl to 2 % was added, as also 0·2 grm. fibrin to both 2 and 3.

After 18 hours in the incubator the fibrin in No. 1 had disappeared: this was not the case in either No. 2 or No. 3, nor had it disappeared after digestion for 24 hours.

This result, in the first instance, confirms the conclusions as to the superior activity of NaCl extracts as compared with aqueous extracts; and, in the second place, it gives an explicit answer to the question propounded above. It clearly shows that the presence of NaCl is of importance in the process of extraction rather than in the process of digestion; and it may be inferred that the NaCl solution dissolved out of the yeast something, no doubt a protease, that water alone failed to extract.

An experiment was made to test the action of a NaCl extract on boiled fibrin. It was found that 60 cc. of a 20 % yeast-extract with NaCl digested 0·2 grm. fibrin in 3 days: the action was slow, but it was more rapid than that of a watery extract of the same strength (see p. 304).

Summary of Experiments on Yeast.

Evidence has been adduced to prove that yeast can effect both peptolysis and peptonization. The fact that these processes can be carried on by filtered extracts makes it clear that they are not due to the yeast-plant as a living organism, but to one or, perhaps, more substances that can be dissolved out of it; and there can be no doubt that the active substance is, in any case, a protease. An important issue is thus raised

that may be expressed in the two questions—(1) is there, as is now generally held, a single protease in yeast, or is there more than one, and in the latter case, how many? (2) What is the nature of the protease or proteases? The results of my experiments will be briefly considered with a view to a reply.

Peptolysis. The most important fact that has been brought to light is the rapidity with which this process is effected: thus in an autolysis-experiment (p. 295) it was found to have proceeded actively in 2½ hours; and in a Witte-peptone experiment (p. 299) in 1 hour. Moreover, in the latter experiment the watery-extract was very dilute (2 %) and the time of extraction very short (1 hour): therefore the protease concerned is readily soluble in water.

It has been shown, further, that peptolysis is most active at or near the natural acidity of the liquid, at a degree of acidity determined by the presence of acid phosphates. It is retarded, and eventually arrested, by any deviation from this degree of acidity, in the direction either of alkalinity or of increased acidity: the effect of added alkali or acid varies with the amount of solid yeast present, or with the strength of the extract, as also with the length of the exposure to its action. Thus, in the case of a 5 % yeast-mixture, peptolysis was found to be inhibited in the presence of either about 0·6 % HCl, or 2 % Na_2CO_3, for 24 hours, as also by exposure to 3 % Na_2CO_3 for 2 hours (p. 296). Similar results were obtained with 5 % watery extracts acting on Witte-peptone (p. 299).

Peptonisation. Under this heading I include the experiments upon the digestion of fibrin.

It has been made clear, in the course of these experiments, that peptonization takes place much less rapidly than peptolysis. Even with relatively strong yeast-extracts several hours were required for the digestion of a small quantity of fibrin: thus 40 cc. of a 5 % yeast-mixture did not digest 0·3 grm. fibrin at all in 22 hours, though the fibrin disappeared within 24 hours more (p. 302).

The next point of importance is the relation between watery extracts of yeast and NaCl extracts. When the extracts were strong (5 % and upwards) and the time of extraction long, the difference in the activity of the two kinds of extracts was not found to be important; but when the extracts were dilute and the time of extraction short, the difference was striking. A rapidly prepared 2 % watery extract did not digest fibrin at all (p. 304), whilst a similarly prepared NaCl extract (p. 305) digested the fibrin in about 24 hours. The inference to be drawn is that the peptonizing enzyme is not readily soluble in distilled water, but is readily soluble in 2 % NaCl solution.

Peptonization was found, like peptolysis, to proceed most actively at or near the natural acidity of the liquid, and to be arrested or retarded by the addition of either acid or alkali. But a comparison of the results

shows that the two processes do not exactly agree in the latter respect: it appears that the range of reaction is rather more limited for peptonization than for peptolysis. Thus, with regard to the action of added acid, the only case in which digestion of fibrin took place in the presence of as much as 0·5 % HCl was one in which the mixture or extract was very strong (20 %, see p. 302); on the other hand, 0·5 % HCl did not inhibit peptolysis in a 5 % yeast-mixture (p. 296) or in 5 % yeast-extract (p. 299). Similarly, with regard to the action of added alkali, whilst it is true that in the experiments in which solid yeast was used, peptonization and peptolysis were equally affected by the addition of Na_2CO_3 to 2 % (compare Expt. 2, p. 302, with Expt. 4, p. 296), there was a marked difference in favour of peptolysis when extracts were employed (compare Expt. 2, p. 303, and Expt. 2, p. 304, with Expts. 2, p. 299, and 4, p. 300). In the two former digestion of fibrin by 10 % or 20 % extracts was inhibited, whilst in the two latter peptolysis was effected by 5 % extracts treated with the same amount of alkali. In Expt. 3, p. 305, digestion of fibrin was inhibited by 1 % Na_2CO_3.

Conclusions. The chief results of the investigation are these:—

(1) dilute yeast-mixtures or aqueous extracts rapidly effect peptolysis, as indicated by the tryptophane reaction, but do not digest fibrin;

(2) dilute NaCl extracts of yeast readily digest fibrin;

(3) peptolysis and peptonization are influenced in the same manner, but not in the same degree, by the addition of acid or alkali.

I infer that these two digestive processes are not effected by one and the same protease. On the contrary, the facts indicate the presence of two proteases: the one exclusively peptolytic, readily soluble in water; the other peptonizing, less soluble in water, but readily soluble in 2 % NaCl solution.

The Mushroom.

Agaricus (Psalliota) campestris.

The discovery of proteases in Basidiomycetous Fungi seems to have been made by Hjort (11), who found that watery extracts of them digested fibrin. In the case of *Agaricus (Pleurotus) ostreatus*, digestion was most active when the liquid was neutral; less active when acidified with 0·5 % oxalic acid, and was altogether inhibited by alkalinity. The fibrin entirely disappeared in 40 hours; the digested liquid then giving no biuret, but strong tryptophane-reaction, and containing leucin and tyrosin. In the case of *Polyporus sulfureus*, the naturally acid extract readily digested fibrin, as did also extracts acidified with HCl to 0·2 % or with oxalic acid to 0·25 %; but neutralized or alkaline extracts did not digest at all. In a 12-hours' digestion the liquid contained albumoses and peptones, but no amido-acids or hexon-bases.

Shortly afterwards the matter was investigated by Bourquelot and Hérissey (12). They found that a filtered watery extract of *Agaricus* (*Amanita*) *muscarius* digested five-sixths of the caseinogen of skim-milk within four days, and they detected tyrosin in the digested liquid. Similar results were obtained with *Polyporus sulfureus*.

In the course of a few experiments with the mushroom, I obtained evidence (13) that the tissue can both peptonize fibrin and peptolyse the lower proteids, thus confirming in a general way the conclusions of my predecessors.

Somewhat more recently the matter has been taken up by Delezenne and Mouton (14), and with widely different results. They prepared extracts, using normal saline solution (0·8 % NaCl) with chloroform or toluol, of the dried pilei of various species (the Mushroom, *Amanita muscaria, Amanita citrina, Hypholoma fasciculare*), which readily peptolysed peptone, and digested gelatine and casein, but had no action on fibrin. This last result seems so contradictory to previous observations that I have thought it necessary to make some further experiments to test its accuracy.

Peptonisation.

The test applied was the disappearance of a relatively small quantity of fibrin. The laminae were in all cases removed from the pileus.

In the first instance the actual tissue, reduced to a pulp, was made use of: provided that the material was mature, the pileus being fully expanded, the result was that digestion of fibrin took place.

Experiment 1. 10 grms. fresh mushroom-pulp were digested with 100 cc. distilled water containing chloroform (0·25 %) and 2 grms. fibrin for 26 hours; at the end of this time the fibrin was completely disintegrated.

Experiment 2. 15 grms. fresh mushroom-pulp were digested with 100 cc. chloroform-water (0·5 %) and 2 grms. fibrin: after 24 hours' digestion the fibrin was dissolved.

Experiment 3. In an experiment similar to and contemporaneous with the preceding, where the antiseptic was HCN (0·2 %), the fibrin was disintegrated and mainly dissolved.

When a watery extract of the fresh ripe pileus was used, from which the solid matter had been removed either by straining through muslin or by filtering through paper, the result was less certain: in most cases the fibrin seemed to be somewhat diminished in quantity, but rapid and complete solution was not constant. The following are some of the more successful experiments:—

Experiment 4. 1 grm. of fibrin was digested with 50 cc. of a watery extract of 40 grms. mushroom-pulp with 200 cc. distilled water, with HCN to 0·2 %: within 24 hours the fibrin was completely disintegrated.

Experiment 5. In this experiment 0·2 grm. of fibrin was completely digested by 50 cc. mushroom-extract, to which NaF to 1 % had been added, in 18 hours.

These results clearly indicated that watery extracts of the Mushroom digest fibrin, in agreement with those of Hjort. However, I was not altogether satisfied, as digestion of fibrin did not occur in every experiment. With the object of obtaining active extracts with greater certainty, I had recourse to the method of extraction with 2 % NaCl solution that had proved serviceable in the case of yeast, and with the same success.

Experiment 6. 120 grms. fresh mushroom-pulp were extracted with 300 cc. 2 % Na Cl solution for 21 hours; the liquid was then strained off through muslin, and placed in 8 bottles each holding 40 cc. with 0·2 grm. fibrin. The treatment of the bottles was—No. 1, nothing added; No. 2, liquid boiled; No. 3, added HCN to 0·2 %; No. 4, added NaF to 1 %; No. 5, added chloroform to 0·5 %; No. 6, added toluol to 0·5 %; No. 7, added HCl to 0·1 %; No. 8, added HCl to 0·2 %.

After 20 hours' digestion in the incubator the fibrin had completely disappeared in Nos. 1, 3, 4, 5, 6; it had not undergone any apparent diminution in Nos. 2, 7, 8.

I found that it is also possible to obtain active extract from dried Mushroom.

Experiment 7. 8 grms. of dried pileus (had been kept for over 6 months) extracted for 4 hours with 100 cc. 2 % NaCl solution; filtered, and added toluol to 1 %. 30 cc. of this liquid had completely digested 0·2 grm. fibrin within 19 hours.

I incidentally observed that 20 cc. of the expressed juice of fresh Mushroom digested 0·2 grm. fibrin within 24 hours in the presence of 1 % toluol.

In another experiment a comparison was instituted between the relative activities of aqueous and 2 % NaCl extracts which were (*a*) of natural acidity, or (*b*) acidified to 0·1 % HCl, or (*c*) made alkaline by adding Na_2CO_3 to 0·5 %, in the presence of toluol.

Experiment 8. 60 grms. fresh mushroom-pulp were extracted for about 24 hours with 250 cc. distilled water; a similar quantity of pulp was extracted for the same time with 250 cc. 2 % NaCl solution. 40 cc. of the filtered aqueous extract were put, with some toluol and 0·2 grm. fibrin, into each of 3 bottles: to No. 1, nothing was added; to No. 2, HCl to 0·1 %; to No. 3, Na_2CO_3 to 0·5 %. 40 cc. of the NaCl extract were also put into each of 3 bottles, and similarly treated.

After 24 hours in the incubator (38–40°C.) the result was that the fibrin had not been digested, or apparently diminished, in any one of the bottles containing the aqueous extract, whilst it had disappeared in the bottle containing the NaCl extract alone, but not in either the Na_2CO_3 or the HCl bottles.

The superior activity of the NaCl extract is more marked, as was the case with yeast, the more dilute the liquids.

Experiment 9. 2·5 grms. of partly dried Mushroom were extracted (4 hours) with 50 cc. 2 % NaCl solution, and an equal quantity with 50 cc. distilled water.

25 cc. of the NaCl extract were put into each of 2 bottles with the addition of some toluol and 0·2 grm. fibrin, the fibrin having been previously boiled in one case: 25 cc. of the watery extract were put, with toluol and unboiled fibrin, into each of 2 bottles, and to one of them 1 grm. NaCl was added.

After 19 hours in the incubator, the unboiled fibrin in the bottle containing NaCl extract had disappeared; the fibrin had not disappeared in any of the other three.

This experiment demonstrates not only the superior activity of the NaCl extract, but also the solvent action of the NaCl (compare Yeast, p. 306).

The next experiment relates to the action of acid and alkali upon a rapidly prepared NaCl extract.

Experiment 10. 100 grms. fresh mushroom-pulp were extracted with 300 cc. of 2 % NaCl solution containing toluol, and filtered, the whole process of preparation occupying about an hour. 40 cc. of the extract were put into each of 5 bottles, with 0·2 grm. of fibrin, treated thus—to No. 1, nothing further added; to No. 2, Na_2CO_3 to 1 %; to No. 3, Na_2CO_3 to 2 %; to No. 4, HCl to 0·1 %; to No. 5, HCl to 0·2 %.

After 24 hours in the incubator, the fibrin had disappeared in No. 1, was unaltered in Nos. 2 and 3, and seemed to be attacked in Nos. 4 and 5: 24 hours later the fibrin had not disappeared in any one of these four bottles.

These results, as also those of Expts. 6 and 8, show that the peptonizing activity of a mushroom-extract, of the strengths employed, is destroyed by the addition to the naturally acid liquid of 0·1 % HCl, or of 0·5–1 % Na_2CO_3.

So far it has been assumed that the disappearance of the fibrin in the experiments implied peptonization. In order that there might be certainty on this point, the following experiment was made:—

Experiment 11. 60 cc. of NaCl extract (toluol 1 %) were put to digest 2 grms. of fibrin: in 20 hours the fibrin had disappeared, and the liquid, after boiling and filtering, gave a well-marked biuret-reaction. At the commencement of the experiment, a sample of the extract gave no biuret-reaction.

The results of these experiments on fibrin are such as to lead inevitably tothe conclusion that the mushroom contains a peptonizing enzyme capable of digesting fibrin: it is therefore remarkable that Delezenne and Mouton (see p. 309) should have expressed the contrary opinion. The reason for this contradiction is that these observers used *boiled* fibrin in their experiments. This precaution, it is true, obviates a possible source of error by eliminating any self-digestion of the fibrin: but it is doubtful if this advantage compensates for the disadvantage involved, the disadvantage of missing altogether the presence of the peptonizing enzyme. Proteids coagulated by heat offer, as is well known, considerable resistance to the digestive action even of animal proteases; so that it is not surprising

that the protease of the mushroom should have failed to act upon them. This precaution is not, however, absolutely indispensable: for it is a simple matter to check possible self-digestion of the fibrin by control-experiments. Thus, in the foregoing Expt. 6, digestion of fibrin took place in Nos. 1, 3, 4, 5, 6, but not in No. 2 where the liquid (but not the fibrin) had been boiled: had the results given by Nos. 1, 3, 4, 5, 6 been due to self-digestion, then the probability is that the same result would have been given by No. 2, which was not the case. All the fibrin used in these experiments was prepared and preserved at one time and in the same manner: hence the fibrin may be regarded as a constant factor, all the variations being due to the mushroom-liquids, as affected by the various substances added to them. I may add that I have not succeeded in observing digestion of boiled fibrin, though strong mixtures and extracts were used, and the experiment was continued for a week.

Peptolysis.

There is already a certain amount of evidence that the mushroom contains an actively peptolytic enzyme, which is to be found in all the papers that I have previously cited (Nos. 11, 12, 13, 14). My main object in making further experiments has been not so much to establish this fact, as to determine the conditions that affect the activity of the protease and so to arrive at some conclusion as to its nature. I may say, however, that I have never failed to obtain from mushrooms, whether ripe or immature, and with great facility, a watery extract—in some cases a glycerine-extract—that readily peptolysed Witte-peptone, as indicated by the tryptophane-reaction. It should be explained that, on account of the deep colour of the liquids, it was not possible to apply the tryptophane-reaction directly: the liquids had first to be boiled with animal charcoal and then filtered.

The following experiment gives a general idea of the method employed and of the results obtained:—

Experiment 1. 90 grms. of fresh mushroom-pulp were extracted for 18 hours with 200 cc. chloroform-water: on straining through muslin, a red, opalescent, acid liquid was obtained.

40 cc. of the liquid were placed in each of 6 bottles, with 0·5 grm. Witte-peptone and a little toluol, the bottles being then treated as follows: added to 1, nothing further; to 2, 1 grm. chalk to neutralize any free acid; to 3, Na_2CO_3 to 1·25 %; to 4, HCl to 0·04 %; to 5, HCl to 0·1 %; to 6, HCl to 0·2 %.

After 23 hours in the incubator the tryptophane-reactions were—1, marked; 2, marked; 3, strong; 4, marked; 5, strong; 6, faint.

There was thus evidence of active peptolysis having taken place within a distinctly alkaline and a distinctly acid range of reaction, and of its arrest in the presence of stronger acid.

The next experiment gives some idea of the rapidity with which peptolysis was found to be effected by a dilute mushroom-extract, and shows further that peptolysis is a much more rapid process than is peptonization.

EXPERIMENT 2. 5 grms. fresh mushroom-pulp were extracted on a filter for about an hour with 100 cc. 1 % toluol-water. The filtered liquid gave no tryptophane-reaction. 50 cc. of it were put into a bottle with 0·3 grm. fibrin, and 50 cc. into another bottle with 0·5 grm. Witte-peptone.

In 1 hour the contents of the Witte-peptone bottle gave a distinct tryptophane-reaction: 24 hours later the reaction was strong. In the same time the fibrin in the other bottle had not disappeared; but it disappeared within the next 24 hours.

The following experiment brings out clearly the relative rapidity of peptolysis and of peptonization, and of the effect of added acid and alkali on these processes respectively:—

EXPERIMENT 3. 5 grms. of dried powdered mushroom were extracted with 200 cc. toluol-water (1 %): 5 grms. were also extracted with 200 cc. of 2 % NaCl solution containing 1 % toluol. 40 cc. of the NaCl extract were put into each of 3 bottles, with 0·2 grm. of fibrin; also 40 cc. into each of 3 bottles with 0·5 grm. Witte-peptone: to 1 fibrin bottle and 1 Witte-peptone bottle (Nos. 1), nothing was added; to another pair of bottles (Nos. 2) Na_2CO_3 to 1 % was added; to a third pair (Nos. 3), HCl to 0·1 % was added. An exactly similar series of bottles containing the aqueous extract was prepared.

After 24 hours' digestion the results were—

	NaCl ext.			*Aq. ext.*		
Nos.	1	2	3	1	2	3
fibrin	gone	not gone	not gone	not gone	not gone	not gone
tryptophane	strong	strong	marked	very strong	strong	strong.

Hence it is apparent that peptolysis and peptonization are independently affected by the addition of acid and alkali.

Conclusions.

Although my investigation of the mushroom has not been so minute as in the case of yeast, the results obtained suffice to draw similar conclusions.

In the first place, the conclusion is justified that the mushroom contains a peptolysing enzyme which is readily extracted by water and acts with rapidity. Secondly, it is equally clear that the mushroom contains a peptonizing enzyme capable of digesting fibrin.

As in the case of yeast, so here, the question arises as to whether both processes are effected by one and the same protease, or whether there are not two proteases in the mushroom, the one especially peptolytic, the other especially peptonizing.

The observed facts are, on the whole, favourable to the latter conclusion. To begin with, the results of the peptolysis-experiment No. 2, suggest that the rapid peptolysis and the slow peptonization should be interpreted as being due to the presence of two proteases, the one readily soluble in water, the other less soluble. Again, the superior peptonizing activity of NaCl extracts compared with watery extracts (see p. 310), suggests that the protease concerned is more readily soluble in 2 % NaCl solution than in distilled water. The importance of NaCl as a solvent is demonstrated in Expt. 9 (p. 310).

The inference drawn from these observations on solubility is supported by the observations upon the effect of added acid and alkali on peptolysis and peptonization respectively. Taking the limits of peptonization as HCl 0·1 % and Na_2CO_3 1 % (p. 311), those of peptolysis are less restricted, extending beyond these limits in both directions.

The Nature of the Proteases.

The generally accepted opinion with regard to the two Fungi in question is that they contain a single protease. In the case of yeast, Hahn and Geret, Bokorny, and others, regard this protease as a trypsin: and Hjort has made the same suggestion in the case of the Basidiomycetous Fungi investigated by him. My observations, as already explained, lead me to the conclusion that two proteases exist in these plants, the one peptolytic, the other peptonizing. It remains now to consider what the nature of these proteases may be.

At a meeting of the Linnean Society of London, on November 20, 1902 (Proceedings, 1902–3, p. 42), I announced the discovery in many plants and different parts of plants of a peptolytic enzyme analogous to the recently discovered entero-erepsin of the animal body: a more complete account of my researches was soon afterwards (January, 1903) published in this periodical (13), the mushroom having been one of the plants investigated.

The further observations, of which an account has now been given, confirm me in the conclusion that the mushroom contains an erepsin, that is, a peptolytic enzyme which is unable to peptonize the higher proteids such as fibrin and albumin; and they justify the extension of the conclusion to yeast.

This vegetable erepsin is not, however, identical in properties with either the entero-erepsin discovered by Cohnheim or the pancreato-erepsin discovered by Vernon (see p. 290). The action of both these animal erepsins is limited to neutral or feebly alkaline liquids, whilst I have found that vegetable erepsin can act through a fairly wide range of acid and alkaline reaction, its greatest activity being manifested when the reaction of the liquid is at or near natural acidity. Hence vegetable erepsin affords a new type of ereptic action.

Now as to the nature of the peptonizing enzyme. It may be either a pepsin or a trypsin, but the question is, which? This question is more easy to put than to answer, because there is at present no method by which the enzyme can be obtained free from the associated erepsin; and until that is done, no direct answer can be forthcoming. But it is possible to form an opinion upon indirect evidence. It should be borne in mind that, as I have elsewhere stated, there is at present no well-established instance of the occurrence of a merely peptonizing enzyme in the Vegetable Kingdom. A more important point is, however, that of the reaction of the liquid in which the protease will work. The activity of animal pepsin is limited to acid liquids; whilst animal trypsin, though most active in a distinctly alkaline liquid, can nevertheless work in a neutral or even in a slightly acid liquid. In its range of reaction, the vegetable peptonizing enzyme resembles animal trypsin rather than pepsin; but with this difference, that whereas animal trypsin is most active in a distinctly alkaline liquid, the vegetable protease is most active in a distinctly acid liquid. It seems therefore probable that the protease in question may be a trypsin of a new type, characterized by its activity in an acid, rather than an alkaline, liquid.

On these grounds it is suggested that the yeast and the mushroom contain two associated proteases, vegetable erepsin and vegetable trypsin, an association that finds its analogue in the pancreatic secretion of animals which, as Vernon has recently shown (2), contains both erepsin (pancreato-erepsin) and trypsin proper. The term 'vegetable trypsin' is already in common use, but in a wider sense than that in which I have just employed it. Hitherto it has been applied to the vegetable proteases without taking the presence of erepsin into account, whereas I limit the term to the peptonizing enzyme apart from the erepsin. Vernon's results have introduced the same distinction into animal physiology; formerly the term 'trypsin' was applied to the protease of the pancreas, but this, as he has shown, is really a mixture of pancreato-erepsin with true trypsin.

A few lines may be devoted, in conclusion, to the consideration of the question as to how far these views are applicable to plants in general. It can hardly be doubted that, at some period in their existence, all plants and all parts of plants contain a peptolytic enzyme concerned in promoting the distribution of proteids in the temporary form of amido-acids, &c. But it is not clear that a peptonizing enzyme is of such general occurrence: on the contrary, as I have already pointed out (13, p. 262), many parts of plants failed to digest fibrin in my experiments. It is possible that in those experiments the precise conditions most favourable to peptonization were not provided: it may be that, for instance, the use of NaCl extracts that have given such good results with the Yeast and the Mushroom, will give similar results in other cases. I have not yet had time to make

extended investigations in this direction; but what already I have done is, I think, of sufficient interest to be mentioned here.

In a previous paper (13, p. 254), I gave an account of some peptonizing experiments with the bulbs of the hyacinth, the tulip, and the onion, the bruised bulb-tissue being employed. The results were not conclusive, but indicated that whilst the onion did not digest fibrin, the hyacinth and the tulip did so to some extent in a slightly alkaline liquid.

I have since resumed these experiments, using watery or NaCl extracts of the bulbs, and taking the disappearance of a small quantity of fibrin as the test of digestion, following the method adopted in the investigation of the yeast and the mushroom, with interesting results.

EXPERIMENT 1. A hyacinth bulb, weighing about 75 grms., was reduced to pulp and extracted for two hours with 100 cc. of 2 % NaCl solution: the liquid was strained through muslin. 30 cc. were put into each of 3 bottles with 0·2 grm. fibrin and some toluol: to No. 1, nothing further was added; to No. 2, HCl to 0·1 %; to No. 3, Na_2CO_3 to 0·5 %.

After 23 hours in the incubator, the fibrin had disappeared in Nos. 1 and 3, and was attacked in No. 2: the tryptophane-reactions were, strong in No. 1, distinct in No. 2, marked in No. 3: 28 hours later the fibrin had disappeared also in No. 2.

EXPERIMENT 2. Similar extracts were prepared of the tulip and onion bulbs: 40 cc. of the extract were in each case put, with 0·2 grm. fibrin, into each of 4 bottles, with the following additions: to No. 1, nothing; to No. 2, HCl to 0·05 %; to No. 3, HCl to 0·2 %; to No. 4, Na_2CO_3 to 1 %.

Within 48 hours the results were: tulip, fibrin gone in No. 1; nearly gone in Nos. 2 and 4; unaltered in No. 3: onion, fibrin unaltered in all. All the bottles gave more or less strong tryptophane-reaction.

The positive results given by the hyacinth and the tulip point to the probability that a peptonizing enzyme is more generally present in plants than is at present recognized. But the negative result given by the onion is even more suggestive; clearly peptolysis (autolysis) had occurred in this experiment without peptonization of the added fibrin. It appears, therefore, that erepsin is present in the onion without any other protease. If this be so, it is important evidence in favour of the existence of an ereptic protease in plants, and strengthens the conclusion, already expressed, that in those plants that can digest fibrin there is also present a distinct peptonizing enzyme.

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3. Butkewitsch: Umwandlung der Eiweissstoffe durch die niederen Pilze, etc.; Jahrb. f. wiss. Bot. xxxviii, 1902, p. 147.
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9. Naegeli: Ernährungschemismus der niederen Pilze; Bot. Mittheilungen, Bd. iii, 1881, p. 464 (Sitzungsber. der K. Bay. Akad. d. Wiss. in München, 5. Juli 1879).
10. ————: Ueber die chemische Zusammensetzung der Hefe; *ibid.* p. 270.
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13. Vines: Proteolytic Enzymes in Plants (I); Ann. Bot., vol. xvii, 1903, p. 254 (January).
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